Major Contribution of the Medial Amygdala to Hypertension in BPH/2J Genetically Hypertensive Mice

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Abstract—BPH/2J mice are recognized as a neurogenic model of hypertension primarily based on overactivity of the sympathetic nervous system and greater neuronal activity in key autonomic cardiovascular regulatory brain regions. The medial amygdala (MeAm) is a forebrain region that integrates the autonomic response to stress and is the only region found to have greater Fos during the night and daytime in BPH/2J compared with BPN/3J mice. To determine the contribution of the MeAm to hypertension, the effect of neuronal ablation on blood pressure (BP) was assessed in BPH/2J (n=7) and normotensive BPN/3J mice (n=7). Mice were preimplanted with radiotelemetry devices to measure 24-hour BP and cardiovascular responses to stress, before and 1 to 3 weeks after bilateral lesions of the MeAm. Baseline BP was 121±4 mm Hg in BPH/2J and 101±2 mm Hg in BPN/3J mice (P<0.001). MeAm lesions reduced BP by 11±2 mm Hg in BPH/2J mice (P<0.001) but had no effect in BPN/3J mice. The hypotensive effect of lesions in BPH/2J mice was similar during both day and night, suggesting that the MeAm has tonic effects on BP, but the pressor response to stress was maintained in both strains. Midfrequency BP power was attenuated in both strains (P<0.05) and the depressor responses to pentolinium after enalaprilat pretreatment was attenuated after lesions in BPH/2J mice (P<0.001; n=3).

These findings indicate that the MeAm provides a tonic contribution to hypertension in BPH/2J mice, which is independent of its role in stress reactivity or circadian BP influences. (Hypertension. 2014;63:811-818). • Online Data Supplement

Key Words: central nervous system ▪ hypertension ▪ stress, physiological ▪ sympathetic nervous system

BPH/2J mice are a genetic model of hypertension developed in the 1970s by Schlager et al.¹ These mice were selectively bred for elevated blood pressure (BP) alongside a normotensive (BPN/3J) and hypotensive control strain (BPL/1J), from a base population of 8 inbred strains of mice. Since then a range of pathophysiological factors have been studied to determine the cause of hypertension in BPH/2J mice, and the relatively modest characterization has been increasing in recent years, possibly because of advances in technology used to study mice.²,³ Our own findings suggest that hypertension in BPH/2J mice is sympathetically mediated, based on the greater depressor response to ganglion blockade, which ultimately abolishes the hypertension in BPH/2J mice and also greater midfrequency mean arterial pressure variability (MAP power).⁴ Even the percentage depressor response to ganglion blockade is greater in BPH/2J mice compared with normotensive controls.⁵ This contrasts findings in spontaneously hypertensive rats (SHRs) that are likely confounded by a vascular amplifier effect⁶ caused by vascular structural changes, which contribute to hypertension in adult SHRs.⁷ Recently, we have also reported that BPH/2J mice have renal sympathetic hyperinnervation and enhanced renin synthesis, which results in a greater contribution of the renin–angiotensin system (RAS) to BP maintenance during the dark period of the 24-hour light cycle in BPH/2J mice compared with BPN/3J mice.⁸ Early studies indicated that BPH/2J mice have aberrant brain catecholamine levels,⁹ suggesting that the hypertension may be centrally mediated. Furthermore, using Fos as a marker of neuronal activity, BPH/2J mice were also shown to have greater neuronal activity in key cardiovascular regulatory brain regions compared with normotensive control BPN/3J mice.⁴ Importantly, of the limbic, hypothalamic, and medullary brain regions analyzed using Fos immunohistochemistry, the medial amygdala (MeAm) was the only region to show greater neuronal activity during the dark (inactive) and the dark (active) period in BPH/2J compared with BPN/3J mice,⁴ suggesting that this region may be inherently overactive in BPH/2J mice. Furthermore, neuronal activity within the MeAm correlated strongly with BP level (R=0.98), suggesting that neuronal activity in the MeAm may be related to BP level.⁴

The MeAm is an important limbic region that integrates sensory information, including stress and fear as well as reproductive-related inputs, and regulates the subsequent cardiovascular, behavioral, and hormonal responses.³⁻¹¹ The MeAm is activated by stressful stimuli particularly of psychological rather than a physiological nature¹² and is involved

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in the integration of olfactory and chemosensory signaling involved in predator and territorial responses and reproduction.\textsuperscript{13,14}\textsuperscript{13} Many studies using Fos as a marker of neuronal activity have shown strong activation of the MeAm associated with stressful stimuli.\textsuperscript{13,15,16} Moreover, inhibition of the MeAm has demonstrated that it does indeed mediate the cardiovascular response to stress in rats, indicating that Fos activity is not merely a response to the stressor.\textsuperscript{17,18} The contribution of the MeAm to baseline BP is not as widely studied. Importantly, Fukumori et al\textsuperscript{19} performed MeAm lesions in prehypertensive 4-week-old SHRs, which attenuated the elevation in BP by 14 weeks. Although there was no normotensive control strain, this study demonstrates that the MeAm contributes modestly to the development of hypertension in SHRs compared with sham controls.

The major aim of the present study was to determine whether there is a causal relationship between the greater neuronal activity within the MeAm and the hypertension or exaggerated stress response in BPH/2J mice. To assess the contribution of the MeAm to hypertension, the effect of MeAm lesions on BP and cardiovascular reactivity to stress was assessed.

**Methods**

**Animals**

Experiments were performed on normotensive BPN/3J (n=13) and hypertensive BPH/2J (n=13) 18-week-old male mice.

**Telemetry Transmitters**

BP telemetry transmitters (model TA11PA-C10; Data Sciences International, St Paul, MN) were implanted as detailed in the online-only Data Supplement.

**Protocol and Experimental Procedures**

After a 10-day recovery period from telemetry surgery, baseline cardiovascular parameters of MAP, heart rate (HR), and locomotor activity were recorded continuously across 48 hours in freely moving mice in their home cage. During the 4 subsequent days, animals were then exposed to a series of behavioral stimuli, including restraint, dirty-cage switch and shaker stress, conducted on separate days during the light period when the animals were inactive, as described previously.\textsuperscript{20,21} Animals underwent the same 48-hour cardiovascular and locomotor recordings and stress test regime 1 and 3 weeks after MeAm lesion or sham surgery. At the conclusion of the study immediately after 1 hour of dirty cage stress, all mice were anesthetized and perfused and brains collected for Fos analysis.

**Lesions of the MeAm**

Lesions were produced in anesthetized BPN/3J and BPH/2J mice (n=7 per group) by performing bilateral microinjections of ibotenic acid into the MeAm. Sham controls underwent the same surgery but no injection was performed (n=6 per group). Details of the lesion surgery are available in the online-only Data Supplement.

**Behavioral Stimuli**

On separate days mice were exposed to 60 minutes of restraint stress, 60 minutes of dirty-cage switch stress, and 5 minutes of shaker stress, as detailed in the online-only Data Supplement.

**Cardiovascular Variability and the Cardiac Baroreceptor Sensitivity**

Spectral analysis of cardiovascular variability and the baroreceptor HR reflex are detailed in the online-only Data Supplement.

**Cardiovascular Response to Angiotensin-Converting Enzyme Inhibition and Ganglion Blockade**

BPH/2J mice (n=3) were administered ganglion blocker, pentolinium (5 mg/kg, IP), 30 minutes after administration of the angiotensin-converting enzyme inhibitor, enalaprilat (1.5 mg/kg, IP). The cardiovascular responses to these drugs were measured at baseline and 3 weeks after MeAm lesions. Angiotensin-converting enzyme inhibition before ganglion blockade has been shown to reduce the compensatory response of the RAS after ganglion blockade, unmasking the full contribution of the sympathetic nervous system (SNS) in these mice.\textsuperscript{7}

**Immunohistochemistry**

Fos immunohistochemistry and analysis were performed on brain sections from mice 4 weeks after sham or MeAm lesion surgery, which were anesthetized and perfused immediately after dirty-cage switch stress. Details on Fos immunohistochemical analysis are available in the online-only Data Supplement.

**Statistical Analysis**

Cardiovascular data were expressed as mean±SEM. The data were analyzed by multi-factor, nested split-plot ANOVA, which allowed for within animal and between animal contrasts.\textsuperscript{22} A combined residual was used that pooled the between and within animal variance as described previously.\textsuperscript{22} A P value of <0.05 was considered significant.

**Results**

**Fos Immunohistochemistry**

Dirty-cage switch stress—induced Fos activation was used to determine the extent of MeAm lesions. Animals were considered to have adequate lesions and were included in analysis only if the extent of the lesion was >60%, as indicated by Fos counts which were <40% of that found in sham controls of each strain. Approximately 60% of mice injected with ibotenic acid were considered to have adequate MeAm lesions. Fos counts in the MeAm of BPH/2J mice with lesions (n=7) were 73% lower than sham controls and in BPN/3J mice (n=7) were 81% lower than sham controls (P<0.001; Figure 1B). Despite Fos activity being markedly reduced in both strains after MeAm lesions, Fos counts were still marginally higher in BPH/2J than in BPN/3J mice (P<0.001; Figure 1B).

Fos counts were 2-fold higher in the central amygdala after MeAm lesions compared with sham in both BPH/2J and BPN/3J (P<0.001, both; Table S1 in the online-only Data Supplement) mice (n=3 per group). Likewise, the Fos counts in the anterior cortical nucleus of the amygdala were greater after MeAm lesions compared with sham in BPH/2J and BPN/3J mice (P<0.001, both; Table S1). However, Fos counts in the basolateral amygdala (BLA) were comparable between BPN/3J mice with MeAm lesions or sham surgery (P=0.1) and were only greater in BPH/2J mice with MeAm lesions compared with sham (P<0.001; Table S1).

**Baseline Cardiovascular Measurements**

MAP in BPH/2J mice was higher than BPN/3J mice during a 24-hour period (P<0.001; n=13 per strain; baseline pooled from sham and lesion groups). During the dark (active) period, MAP in BPH/2J mice was 25% greater, HR was 37% greater, and locomotor activity was 4.4-fold greater than BPN/3J mice (P<0.001 all). During the light (inactive) period, MAP in BPH/2J mice was 12% greater than in BPN/3J mice...
(P<strain=0.04) and HR was 21% greater (P<strain=0.002), but locomotor activity was comparable between strains (P<strain=0.9).

Effect of MeAm Lesions on Cardiovascular and Locomotor Measurements

After MeAm lesions, 24-hour MAP was reduced in BPH/2J mice by –10.3±2.8 mm Hg after 1 week (P<lesion<0.001) and by –12.6±4.2 mm Hg after 3 weeks (P<lesion<0.001; Table 1; n=7). The hypotensive effect of MeAm lesions in BPH/2J was similar at 1 and 3 weeks post lesion (P<week=0.7) and was also similar during the light (inactive) and dark (active) periods (P<state>0.1; Figure 2A). By contrast, BPN/3J mice showed no change in MAP at 1 or 3 weeks after MeAm lesion (P<lesion>0.8; P<week=1.0; n=7). Furthermore, 1 and 3 weeks after sham surgery, MAP was comparable with baseline in BPN/3J or BPH/2J mice (P<sham>0.3; Table 2; Figure 2B; n=6 per group).

The hypotensive effect of MeAm lesions was markedly different from that in BPN/3J mice (P<strain=0.002) and also different from the effect of sham surgery in BPH/2J mice (P<0.03). Furthermore, the elevated MAP during the light (inactive)

period at baseline in BPH/2J compared with BPN/3J mice was abolished after MeAm lesions (P<strain=0.8) although MAP during the dark (active) period still remained greater in BPH/2J mice (P<strain=0.001).

MeAm lesions reduced HR in BPH/2J mice at 1 and 3 weeks post lesion (P<lesion<0.03; Tables 1 and 2) but HR was not changed in BPN/3J mice (P<lesion>0.6). Likewise, after sham surgery, HR was lower by 3 weeks in BPH/2J mice (P<sham<0.001) but HR was not changed in BPN/3J mice (P<sham>0.3). However, the mild bradycardic effect of MeAm lesions in BPH/2J mice was similar to the effect of MeAm lesions in BPN/3J mice (P<strain=0.3) and also similar to the effect of sham surgery in BPH/2J mice (P<strain=0.8). After MeAm lesions there was no strain difference in HR during the light (inactive) period (P<strain=0.1) but HR still remained markedly elevated in BPH/2J mice compared with BPN/3J mice during the dark (active) period (P<strain<0.001; Figure S1). MeAm lesions or sham surgery did not affect locomotor activity in BPN/3J or BPH/2J mice at 1 or 3 weeks after lesion (P<lesion>0.8; P<sham>0.5; Tables 1 and 2).

Table 1. Average 24-hour MAP, HR, and Locomotor Activity at Baseline and 1 and 3 Weeks After Medial Amygdala Lesions in BPN/3J and BPH/2J Mice

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Baseline</th>
<th>Week 1 PL</th>
<th>Week 3 PL</th>
<th>Baseline</th>
<th>Week 1 PL</th>
<th>Week 3 PL</th>
</tr>
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<tr>
<td>MAP, mm Hg</td>
<td>102±3</td>
<td>103±2</td>
<td>104±2</td>
<td>121±5*</td>
<td>110±6†</td>
<td>108±6†</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>407±21</td>
<td>421±16</td>
<td>416±17</td>
<td>517±27*</td>
<td>486±23‡</td>
<td>483±23‡</td>
</tr>
<tr>
<td>Activity, U</td>
<td>0.4±0.1</td>
<td>0.6±0.1</td>
<td>0.7±0.2</td>
<td>1.8±0.3*</td>
<td>1.5±0.3</td>
<td>1.8±0.6</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>31.5±0.8</td>
<td>30.3±0.6</td>
<td>31.3±0.8</td>
<td>24.8±0.4§</td>
<td>25.2±0.4</td>
<td>25.4±0.4</td>
</tr>
</tbody>
</table>

Values are mean±SEM. bpm indicates beats per minute; MAP mean arterial pressure; and PL post lesion. Comparison of baseline with 1 and 3 wk post lesion in BPN/3J and BPH/2J represented by †P<0.001 and ‡P<0.05. Between strain comparison of baseline measurements is represented by *P<0.001 and §P<0.01.

Figure 1. A, Bar graph represents mean±SEM Fos counts induced by dirty-cage switch stress throughout regions of the medial amygdala (MeAm) in sham (filled) and lesion mice (BPN/3J and BPH/2J mice combined; hatched). B, Means±SEM Fos counts in BPN/3J (gray) and BPH/2J mice (black) in sham (unfilled) and lesion mice (filled). Comparison between sham and lesion in each strain represented by ***P<0.001. Comparison between BPN/3J and BPH/2J mice represented by ▲P<0.05 and ▲▲P<0.001. C, Photomicrograph depicts dirty-cage switch stress-induced Fos activity within the MeAm of representative BPH/2J mice that have undergone sham surgery (left) and MeAm lesion surgery (right) 3 weeks earlier. Opt indicates optic tract.
Cardiovascular Variability and Cardiac Baroreflex Sensitivity

During the light (inactive) period, midfrequency MAP was 33% greater in BPH/2J than in BPN/3J mice at baseline ($P_{\text{strain}}=0.04$) and midfrequency HR power and baroreflex gain were also greater in BPH/2J mice ($P_{\text{strain}}<0.001$; baseline pooled from sham and lesion groups). Measurements from 1 and 3 weeks after sham or lesion were combined. Midfrequency MAP power was markedly reduced in both strains after MeAm lesions ($P_{\text{lesion}}<0.01$) but was not effected by sham surgery ($P_{\text{sham}}>0.2$). Midfrequency HR power was not affected by MeAm lesions in either strain ($P_{\text{lesion}}<0.01$) but was markedly augmented after sham surgery ($P_{\text{sham}}<0.001$). Baroreflex gain was increased in both strains after sham surgery ($P_{\text{sham}}<0.01$) and MeAm lesions ($P_{\text{lesion}}<0.001$).

During the dark (active) period, midfrequency MAP power was 74% greater in BPH/2J than in BPN/3J mice ($P_{\text{strain}}<0.001$), whereas midfrequency HR power and baroreflex gain were similar between strains (Figure 3, bottom; $P_{\text{strain}}=0.08$). MeAm lesions reduced midfrequency MAP power by 46% in both BPH/2J ($P_{\text{lesion}}<0.001$) and BPN/3J mice ($P_{\text{lesion}}=0.02$) and resulted in similar postlesion midfrequency MAP power between strain ($P_{\text{strain}}=0.1$). Moreover, sham treatment had no effect on midfrequency MAP power in either strain ($P_{\text{sham}}>0.2$). Midfrequency HR power was increased after MeAm lesion in BPH/2J ($P_{\text{lesion}}<0.001$) but not BPN/3J mice ($P_{\text{lesion}}=1.0$), and sham surgery had no effect in either strain ($P_{\text{sham}}>0.2$). Baroreflex gain was augmented in each strain after MeAm lesions ($P_{\text{lesion}}<0.001$) but unchanged after sham surgery ($P_{\text{sham}}=1.0$).

Cardiovascular Response to Angiotensin-Converting Enzyme Inhibition and Ganglion Blockade

Treatment with enalaprilat reduced MAP from baseline in BPH/2J during the dark period ($-11\pm2$ mmHg; $P<0.001$; $n=3$) but not during the light period ($+3\pm3$ mmHg; $P=0.25$; $n=3$; Figures 4 and S2). MeAm lesions abolished the enalaprilat-induced depressor response during the dark period in BPH/2J ($P_{\text{lesion}}<0.04$; $n=3$) and did not influence the effect of enalaprilat during the light period ($P_{\text{lesion}}<0.6$; $n=3$).

After pretreatment with enalaprilat in BPH/2J, intraperitoneal injection of pentolinium caused marked depressor responses during the light ($-54\pm2$ mmHg; $n=3$) and dark periods ($-50\pm2$ mmHg; $n=3$; Figure 4 and S3), which were in addition to the effect of enalaprilat. After MeAm lesions, the depressor responses to pentolinium were attenuated by 29% and 27% during the light and dark periods, respectively ($P_{\text{lesion}}<0.05$).

Cardiovascular Response to Behavioral Tests

Dirty-cage switch stress induced rises in MAP, which were 45% greater in BPH/2J compared with BPN/3J mice at baseline.
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This was accompanied by increased locomotor activity that was 1.9-fold greater in BPH/2J mice ($P_{\text{strain}}<0.001$) and tachycardic responses, which were comparable between strains ($P_{\text{strain}}=1.0$). Cardiovascular responses to stress measured 1 and 3 weeks after sham or lesion surgery were combined. The pressor response to dirty-cage switch in BPH/2J mice was augmented by 14% after MeAm lesions ($P_{\text{lesion}}=0.052$) but was attenuated by 12% after sham surgery ($P_{\text{sham}}=0.05$; Figure 5). This resulted in a time by treatment interaction ($P_{\text{Int}}<0.001$). The pressor response induced by dirty-cage switch was unaffected by MeAm lesion or sham surgery in BPN/3J mice ($P_{\text{lesion}}=1.0$; $P_{\text{sham}}=0.2$). Tachycardic and locomotor activity responses to dirty-cage switch were not influenced by MeAm lesion or sham surgery in either strain ($P_{\text{lesion}}>0.5$; $P_{\text{sham}}>0.6$).

Pressor and tachycardic responses induced by 2 other aversive stressors, 1 hour of restraint stress and 5 minutes of shaker stress, were unaffected by MeAm lesion or sham surgery in BPN/3J and BPH/2J mice ($P_{\text{lesion}}>0.2$; $P_{\text{sham}}>0.1$; Figures S4 and S5).

**Figure 3.** Left, Average midfrequency (MF, 0.3–0.5 Hz) mean arterial pressure (MAP) power (mmHg$^2$); middle, MF HR power (bpm$^2$); right, baroreflex gain from cross-spectral analysis (gain, bpm/mmHg) during the light (inactive) phase (top), and dark (active) phase (bottom). Measurements are at baseline (B, filled bars; baselines pooled from sham and lesion groups) and after sham surgery (S, hatched bars) and MeAm lesion surgery (L, unfilled) in BPN/3J (gray; n=6–7) and BPH/2J mice (black; n=6–7). Post sham and lesion values are an average of measurements at 1 and 3 weeks post surgery. Values are means±SEM. Effect of sham or MeAm lesions compared with baseline in each strain *$P<0.05$; **$P<0.01$; and ***$P<0.001$. Between strain comparison of baseline measurements †$P<0.05$ and †††$P<0.001$.

**Figure 4.** Mean arterial pressure (MAP) response to administration of enalaprilat (top) and pentolinium after pretreatment with enalaprilat (bottom) in BPH/2J (black) mice during the light (inactive) period (left) and dark (active) period (right). Line graphs include measurements at baseline (filled circles) and after MeAm lesion surgery (open squares). Each point represents the mean value averaged across a 5-minute period. The dashed vertical reference line represents the time point of administration of treatment. Bar graphs represent mean±SEM change in MAP in response to enalaprilat (top) and the subsequent response to pentolinium (bottom) after enalaprilat pretreatment in BPH/2J mice at baseline (B) and after medial amygdala (MeAm) lesions (L). Significance refers to effect of MeAm lesions *$P<0.05$ and **$P<0.01$. 

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*Figure 3. Left, Average midfrequency (MF, 0.3–0.5 Hz) mean arterial pressure (MAP) power (mmHg$^2$); middle, MF HR power (bpm$^2$); right, baroreflex gain from cross-spectral analysis (gain, bpm/mmHg) during the light (inactive) phase (top), and dark (active) phase (bottom). Measurements are at baseline (B, filled bars; baselines pooled from sham and lesion groups) and after sham surgery (S, hatched bars) and MeAm lesion surgery (L, unfilled) in BPN/3J (gray; n=6–7) and BPH/2J mice (black; n=6–7). Post sham and lesion values are an average of measurements at 1 and 3 weeks post surgery. Values are means±SEM. Effect of sham or MeAm lesions compared with baseline in each strain *$P<0.05$; **$P<0.01$; and ***$P<0.001$. Between strain comparison of baseline measurements †$P<0.05$ and †††$P<0.001$.

Figure 4. Mean arterial pressure (MAP) response to administration of enalaprilat (top) and pentolinium after pretreatment with enalaprilat (bottom) in BPH/2J (black) mice during the light (inactive) period (left) and dark (active) period (right). Line graphs include measurements at baseline (filled circles) and after MeAm lesion surgery (open squares). Each point represents the mean value averaged across a 5-minute period. The dashed vertical reference line represents the time point of administration of treatment. Bar graphs represent mean±SEM change in MAP in response to enalaprilat (top) and the subsequent response to pentolinium (bottom) after enalaprilat pretreatment in BPH/2J mice at baseline (B) and after medial amygdala (MeAm) lesions (L). Significance refers to effect of MeAm lesions *$P<0.05$ and **$P<0.01$. 

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(P $\text{strain}<0.001$; baseline pooled from sham and lesion groups).
Discussion

The main finding from the present study was that MeAm lesions caused marked reductions in BP in BPH/2J hypertensive mice, which abolished the BP difference between BPN/3J and BPH/2J mice during the light (inactive) period and reduced the BP difference by more than half during the dark (active) period. The MeAm lesions did not affect BP in BPN/3J mice, suggesting that the activity of neurons within the MeAm is causally associated with hypertension but may not be critical for maintenance of BP in normotensive mice. The hypotensive effect of MeAm lesions in BPH/2J mice was similar during both the inactive and active periods, suggesting that the MeAm contains neurons that exert a tonic effect on BP, independent of the animal’s state. MeAm lesions also reduced midfrequency MAP power in both strains, suggesting that the activity of neurons in the MeAm may influence SNS outflow. The depressor response induced by ganglion blockade was also attenuated in BPH/2J mice after MeAm lesions, supporting the notion that the MeAm may influence the SNS. Finally, MeAm lesions did not reduce the pressor response to any of the stressors in BPH/2J mice, suggesting that the hypotensive effect of MeAm lesions is not a consequence of attenuated stress reactivity. Together these results suggest that the MeAm is contributing to the hypertension in BPH/2J mice possibly via tonic activation of the SNS, which is independent of its role in stress reactivity and circadian BP influences.

The hypotensive effect of MeAm lesions in BPH/2J mice is consistent with studies in SHRs, which showed that lesions of the entire amygdala or neurons specifically within the MeAm of SHRs at an early age attenuate the development of hypertension. Galeno et al. also suggested that the central amygdala contributes to hypertension in SHRs, but further examination of this hypothesis revealed that this was likely because of an effect on reduced body weight gain rather than a specific effect on hypertension, which is independent of phasic changes in BP associated with the circadian cycle. Taken together, these studies suggest that neurons within the MeAm normally have little influence on long-term levels of BP but make a considerable contribution to the development and maintenance of hypertension in ≥2 genetic models, namely SHRs and BPH/2J mice.

We have reported previously that neuronal activity within the MeAm, as indicated by Fos expression, correlates strongly with the depressor response to ganglion blockade in BPH/2J and BPN/3J mice, suggesting that neuronal activity in the MeAm may be related to the contribution of the SNS to BP level. The reduction in midfrequency MAP power in both strains after MeAm lesions suggests that the MeAm may indeed influence SNS activity and this was further supported by the attenuation of the depressor response to ganglion blockade apparent in a cohort of BPH/2J mice. Therefore 2 indicators of SNS activity suggest that neurons within the MeAm influence on BP in rodents. The MeAm has been demonstrated previously to mediate phasic changes in cardiovascular parameters during stress, chemoreflex, and baroreflex activation. However, acute inhibition of the MeAm in normotensive rats has consistently demonstrated no effect on resting BP, indicating that neurons in this region are unlikely affecting tonic levels of BP. The lack of effect of MeAm lesions on BP in BPN/3J mice supports these prior findings in normotensive animals but the same was not true in hypertensive BPH/2J mice. It is interesting to note that there were greater Fos counts in the BLA after stress in BPH/2J mice with MeAm lesions but not in BPN/3J mice. One might suggest that the hypotensive effect of MeAm lesions in BPH/2J mice may be secondary to changes in the neuronal activity in the BLA. However, electric stimulation of the BLA in conscious rats and pharmacological dis-inhibition of the BLA in anesthetized rats can cause increases in BP. Therefore, it seems unlikely that increased neuronal activity within the BLA is driving the hypotensive effect of MeAm lesions in BPH/2J mice. Another surprising finding was that comparable reductions of BP in BPH/2J mice occurred across the entire 24-hour period, which suggests that the contribution of the MeAm to elevated BP is likely mediated by a tonic pressor influence, which is independent of phasic changes in BP associated with the circadian cycle. Taken together, these studies suggest that neurons within the MeAm normally have little influence on long-term levels of BP but make a considerable contribution to the development and maintenance of hypertension in ≥2 genetic models, namely SHRs and BPH/2J mice.

Figure 5. Line graph represent average mean arterial pressure (MAP), heart rate (HR, bpm), and locomotor activity (Act) responses before and during dirty cage switch stress in BPN/3J (gray) and BPH/2J mice (black) at baseline (baselines pooled from sham and lesion groups). Each dot represents mean±SEM, averaged across a 10-minute period. Bars graphs represent average MAP, HR, and locomotor activity response to the stimuli at baseline (B) and average response after sham surgery (S) or medial amygdala lesion (L). Post sham and lesion values are an average of measurements at 1 and 3 weeks post surgery. Values are mean±SEM. *P<0.05 represents comparison of post-treatment responses with baseline responses in each strain.
the SNS although these measures cannot rule out postsynaptic
effects, and direct sympathetic nerve recording would be
necessary to definitively confirm an influence of the MeAm
on sympathetic outflow. In addition, MeAm lesions abolish
the depressor response to angiotensin-converting enzyme inhibi-
tion during the dark period in BPH/2J mice, suggesting that
the MeAm also influences BP in BPH/2J mice via actions on
the RAS, presumably through neurally (sympathetic) medi-
ated renin release. This is consistent with our recent findings,
which suggest that the sympathetic activation of the renal RAS
likely contributes to the hypertension in BPH/2J mice. Indeed,
if the MeAm does influence BP in BPH/2J mice via sympa-
thetically mediated enhancement of the RAS, this could poten-
tially explain why MeAm lesions reduced midfrequency MAP
power in both strains but only reduced BP in BPH/2J mice.

One unexpected finding was the lack of effect of MeAm
lesions on the acute cardiovascular response to stress or arousal.
Dirty-cage switch stress did induce marked neuronal activa-
tion in sham mice, consistent with increased neuronal activity
measured after many different types of stressful stimuli. Interestingly, despite dirty-cage switch–induced Fos activity
being markedly lower in mice with MeAm lesions, the cardio-
vascular response was unaltered, suggesting that the MeAm
may not be essential for producing the cardiovascular response.
However, the MeAm has been demonstrated previously to
influence the cardiovascular response to stress, as inhibition
of the MeAm with a γ-aminobutyric acidA agonist muscimol
attenuated pressor responses induced by restraint stress in
rats by approximately one third. Thus, the MeAm certainly
seems capable of influencing the magnitude of the cardiovas-
cular response to stress. Yet, the reason for a lack of effect
of lesions on cardiovascular response to stress in the present study
is unclear. One possibility is that sufficient neuronal activity
in the MeAm may have remained after lesions such that the
stress-mediating function of the MeAm was preserved. In the
present study, the extent of lesions was determined by a func-
tional assessment of stress-induced neuronal activation. To this
end, we found that lesions reduced Fos activation induced by
exposure to stress by >70%. Thus, if the relatively few remain-
ing neurons were adequate to maintain the cardiovascular
response, this would indicate a high level of redundancy within
the MeAm. Alternatively, adaptive neuronal remodeling may
occur in the weeks after lesions, such that other brain regions
assume greater regulatory control over the stress response, per-
haps because of a high degree of functional redundancy within
the extended amygdala. Indeed, preliminary findings show
greater Fos counts in the anterior cortical nucleus of the amygd-
ala and central amygdala after dirty-cage switch stress in mice
with MeAm lesions compared with sham animals. These find-
ings suggest that neuronal activity within the extended amygd-
ala was altered by lesions of the MeAm. However, whether the
increased neuronal activation of other amygdaloid regions
occurs only transiently in response to stress after MeAm lesions
or whether this is a change independent of stress is unclear.
To investigate this finding more extensively in the future, mea-
surement of longer lasting immunohistochemical indicators of
neuronal activity would need to be assessed in nonstressed
mice with and without MeAm lesions. However, regardless of
the reason for the lack of effect of MeAm lesions on the stress
response, these findings indicate that phasic BP responses were
unaffected by MeAm lesions. Thus, the hypotensive effect of
MeAm lesions in BPH/2J mice was not caused by reductions in
acute cardiovascular responses to stress or arousal.

Perspectives
The results of the present study demonstrate that neurons
located within the MeAm contribute to elevated BP in hyperten-
sive BPH/2J mice, suggesting that altered function within this
region makes a profound contribution to the hypertension. To
our knowledge, no study has explored directly the association
between the amygdala and hypertension in humans. However,
clinically there is an association between amygdala activity with
exaggerated pressor responses to stress, which in turn is associ-
ated with greater risk of developing hypertension. Hypertension
in BPH/2J mice has been likened to that of white-coat hyper-
tensive patients based on SNS hyper-responsivity in addition to
exaggerated circadian-related BP surges and cardiovascular
hyper-reactivity to stressful situations shown in both BPH/2J
mice and white-coat hypertensive patients. Given that the
present study demonstrates a substantial contribution from the
MeAm to maintenance of the hypertensive state in BPH/2J mice,
this may prompt more extensive investigation into the role of this
and possibly other forebrain regions, which have been suggested
to be a major cause of hypertension in humans.

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Disclosures
None.

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concentrations in discrete brain nuclei and sympathetic tissues of geneti-
The role of the medial and central amygdala in stress-induced suppression


Novelty and Significance

What Is New?

• The medial nucleus of the medial amygdala contributes to the established hypertension in BPH/2J mice but not blood pressure maintenance in normotensive control mice.

What Is Relevant?

• The present study demonstrates a substantial contribution of a relatively understudied limbic brain region to a genetic/neurogenic model of hypertension.
Major Contribution of the Medial Amygdala to Hypertension in BPH/2J Genetically Hypertensive Mice
Kristy L. Jackson, Kesia Palma-Rigo, Thu-Phuc Nguyen-Huu, Pamela J. Davern and Geoffrey A. Head

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MAJOR CONTRIBUTION OF THE MEDIAL AMYGDALA TO HYPERTENSION IN BPH/2J GENETICALLY HYPERTENSIVE MICE

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Disclosures: none

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Supplement Materials and Methods

Animals
The genetically hypertensive BPH/2J mice (n=13) and normotensive BPN/3J (n=16) male mice used in the present study came from inbred colonies bred at the Alfred Medical Research and Education Precinct Animal Centre (Generation 15-20) from breeders purchased at generation 20-36 from Jackson laboratories. The original breeding selection program, took place in the 1970’s for at least 23 generations and then brother sister mating followed to create these inbred strains. All mice used in the present study were housed in individual cages with environmental enrichment, in a room with a 12:12 hour light-dark cycle (1am–1pm light/day) with *ad libitum* access to water and mouse chow (Specialty Feeds, Glen Forrest, Western Australia; 19% protein, 5% fat, 5% fibre, 0.2% sodium). The experiments were approved by the Alfred Medical Research Education Precinct Animal Ethics Committee and conducted in accordance with the Australian Code of Practice for Scientific Use of Animals, in line with international standards.

Radiotelemetry transmitter implantation
Blood pressure (BP) telemetry transmitters (model TA11PA-C10; Data Sciences International, St Paul, MN) were implanted under isoflurane open circuit anesthesia (5% induction and 1.5-2% maintenance) (Forthane, Abbott, Botany, NSW, Australia) delivered via oxygen. Carprofen (5mg/kg)(Rimadyl, Pfizer Australia Pty Ltd, West Ryde, NSW, Australia) was administered subcutaneously just prior to surgery and 24 hours post-surgery for analgesia. A lateral incision and blunt dissection were used to expose the left carotid artery which was temporarily occluded using a non-absorbable silk tie (Dysilk 1-0, Dynek Pty Ltd, SA, Australia). The catheter of the telemetry device was inserted into the carotid artery and secured using silk ties and the body of the probe was positioned subcutaneously along the right flank. A subcutaneous continuous stitch using an absorbable suture (Polysorb, Covidien, Mansfield, MA) was used to close the incision. Mice were allowed at least ten days recovery prior to BP measurement. Cardiovascular and locomotor recordings were sampled at 1000 Hz using an analog-to-digital data acquisition card (National Instruments 6024E) as described previously.

Lesions of the medial amygdala
Mice were anaesthetized with a combination of 100mg/kg Ketamine (Ketalar, Pfizer), 10mg/kg Xylazine (Ilium Xylazil-20, Smithfield, Australia) and 1.2mg/kg Atropine
Mice were placed in the prone position in a stereotaxic apparatus (Angle Two™, Leica, USA) the skull exposed and holes (~1mm) drilled into the skull in the appropriate locations. Four bilateral microinjections (~30nl each) of 10ug ibotenic acid/ 1ul 0.1M NaOH (Tocris bioscience, Bristol, UK) were made into the MeAm via a glass micropipette (~100um tip), connected to a micromanipulator via SV 10 tubing, directed by the computer aided stereotaxic apparatus. Sham controls underwent the same surgery where the micropipette was lowered to the same co-ordinates but no injection performed. Coordinates from bregma: medial/lateral (ML) +/-1.9mm mm, anterior/posterior (AP) -1.7mm mm dorsal/ventral (DV) -5.4mm; ML +/-2.2mm, AP -1.7mm, DV -5mm; ML +/-2.05mm, AP -1.82mm, DV -5.4mm; ML +/-2.05mm, -1.58mm, DV -5.4mm. Post-operative analgesia was provided by subcutaneous administration of 5mg/kg Carprofen (Pfizer Australia Pty Ltd, West Ryde, NSW, Australia).

**Behavioural stimuli**

Dirty cage-switch stress involves placing mice in a cage previously occupied by another male mouse. This stressor was also conducted for 60 minutes and has been demonstrated to induce sustained pressor responses as well as increased locomotor activity. The dirty cages used for this stress were generally occupied by a male mouse of a different strain for approximately a week beforehand. All behavioural tests are conducted before lesions and following 1 and 3 weeks post lesion. As such the stressors are not considered to be novel when conducted post lesion. Shaker stress involves placing the telemetry receiver and mouse in their home cage onto an orbital shaker (Ratek, Baronia, Victoria, Australia) at a speed of 90 rotations per min for 5 min. Restraint stress involved guiding the mouse into a cylindrical Plexiglas restrainer with a sliding back plate to confine the animal for 60 minutes. Immobilisation minimises the contribution of locomotor activity to cardiovascular response to stress.

**Fos Immunohistochemical Analysis**

Mice were deeply anaesthetized with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg) following 1-hour of dirty cage swap stress, conducted during the light (inactive) period. Animals were perfused transcardially with 20 ml of 0.9 % saline and 60 ml of 4 % paraformaldehyde dissolved in 0.1 M phosphate buffer, pH 7.2 (PB). Subsequently, the brain was removed and postfixed for 1-hour in 20 % sucrose in paraformaldehyde, and placed in 20 % sucrose in PB and refrigerated overnight at approximately 4 °C. Coronal sections (40 µm) were cut on a cryostat and
placed in PB. Free-floating sections were incubated in 10 % normal horse serum at room temperature for 1-hour. Sections were then incubated in primary antibody, sheep anti-c-Fos (Chemicon) diluted 1:2000 in a solution of 2 % normal horse serum and 0.3 % Triton X-100 (Sigma) in PB at room temperature overnight. Sections were washed in PB prior to incubation in biotinylated donkey anti-sheep immunoglobins (1:200, Jackson) in PB containing 2 % normal horse serum for 1-hour. Thereafter, the sections were washed and incubated in avidin-biotin peroxidase complex (1:100, Vector) in PB for 1-hour. Following washes in 0.05 M Tris buffer (pH 7.6), sections were incubated in a solution of 40 mg nickel ammonium sulphate and 50 mg 3-3′diaminobenzidine hydrochloride per 100 ml Tris buffer for 10 mins, 15 µl of 30 % hydrogen peroxide was added for a further 6-mins. Following final washes, sections were mounted on gelatin coated microscope slides. Bright-field illumination using a Motic BA400 microscope and Motic images plus 2.0 were used to assess the Fos-immunoreactivity in the MeAm as detected by black stained nuclei. Fos staining was counted in two brain sections per coronal atlas plate between -0.94-2.06mm from bregma, within the boundaries of the MeAm as outlined in the mouse brain atlas.5,6

**Cardiovascular variability and the cardiac baroreceptor sensitivity**

Beat-to-beat data were analysed separately to calculate power spectra using a program written in Labview.7 The auto- and cross-power spectra were calculated for multiple overlapping (by 50%) segments of MAP and HR using a Fast Fourier transform as adapted for conscious mice.8 The cardiac baroreflex sensitivity was estimated as the average value of the transfer gain in the frequency band between 0.3 and 0.5 Hz.8 Baroreflex slope was considered significant if the coherence between MAP and HR across several overlapping segments in the analysed frequency band was >0.4. Data periods with low locomotor activity were chosen (4 from each circadian period) from 48 hour recordings minimizing the influence of physical activity.

**Cardiovascular response to ACE inhibition and ganglion blockade**

Following a 30 minute baseline, BPH/2J mice (n=3) were administered with an ACE inhibitor, enalaprilat (1.5mg/kg, IP) followed 30 minutes later by the ganglion blocker, pentolinium (5mg/kg, IP).9 To calculate the effect of enalaprilat, the 30 minute control period immediately preceding the enalaprilat injection was compared with the response 15-30 minutes following enalaprilat injection. To calculate the effect of pentolinium, the 15 minute period immediately preceding the pentolinium injection was used as the
control period and compared with the response 15-30 minutes following the pentolinium injection.

References


Supplementary results

Table S1: Fos counts in the anterior cortical amygdala, basolateral amygdala and central amygdala in BPN/3J and BPH/2J mice following stress in mice with sham or medial amygdala lesions.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>BPN/3J</th>
<th>BPH/2J</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>MeAm lesion</td>
</tr>
<tr>
<td>ACo</td>
<td>8.4 ± 0.9</td>
<td>15.8 ± 1.3</td>
</tr>
<tr>
<td>BLA</td>
<td>13.3 ± 1.3</td>
<td>15.4 ± 0.6</td>
</tr>
<tr>
<td>CeAm</td>
<td>4.7 ± 0.8</td>
<td>9.3 ± 0.6</td>
</tr>
</tbody>
</table>

Anterior cortical nucleus of the amygdala (ACo), basolateral amygdala (BLA), central amygdala (CeAm). P value represents a within strain comparison of counts in mice following medial amygdala (MeAm) lesions compared with sham.
**Figure S1:** Line graphs show hourly averages of mean arterial pressure (MAP), heart rate (HR, beats per minute) and locomotor activity (Act) during the dark (active, outer panels) and light (inactive, middle panel) phases in BPN/3J (Left, grey n=7) and BPH/2J (Right, black, n=7). Line graphs include measurements at baseline (pretreatment, open circles) and following A; MeAm lesion surgery and B; sham surgery (average of 1&3 weeks post lesion, closed circles). Histograms are mean change ± SEM from baseline post lesion during the light (inactive) period (unfilled) and dark (active) period (hatched) in BPN/3J (grey) and BPH/2J (black). Difference from baseline *P<0.05; **P<0.01; ***P<0.001.
Figure S2: Mean arterial pressure (MAP), heart rate (HR) and locomotor activity response to administration of enalaprilat in BPH/2J (black) mice during the light inactive period (left, n=3) and dark active period (right, n=3). Each point represents the mean value averaged across a 5-minute period. The dashed vertical reference line represents the time-point of administration of treatment. Shaded area represents the period analyzed for comparison of the effect of treatment. Bar graphs represent average changes in MAP in response to agents in BPH/2J mice at baseline (B) and after MeAm lesions (L). Bar graphs values are mean±SEM. Significance refers to effect of lesion *P<0.05.
Figure S3: Mean arterial pressure (MAP), heart rate (HR) and locomotor activity response to administration of pentolinium following pre-treatment with enalaprilat in BPH/2J (black) mice during the light inactive period (left, n=3) and dark active period (right, n=3). Each point represents the mean value averaged across a 5-minute period. The dashed vertical reference line represents the time-point of administration of treatment. Shaded area represents the period analyzed for comparison of the effect of treatment. Bar graphs represent average changes in MAP in response to agents in BPH/2J mice at baseline (B) and after MeAm lesions (L). Bar graphs values are mean±SEM. Significance refers to effect of lesion *P<0.05, **P<0.01.
Figure S4: Line graph represent average mean arterial pressure (MAP) and heart rate (HR, beats per minute) responses before and during restraint stress at baseline in BPN/3J (grey) and BPH/2J mice (black) (Baselines pooled from sham and lesion groups). Each dot represents mean ± SEM averaged across 10-minute periods. Bar graphs represent average MAP and HR response to the stimuli at baseline (B) and average response following sham surgery (S) or MeAm lesion (L). Post sham and lesion values are an average of measurements at 1 and 3 weeks post-surgery. Values are mean ± SEM. *, P<0.05 represents comparison of post-treatment responses with baseline responses in each strain.
Figure S5: Line graph represent average mean arterial pressure (MAP), heart rate (HR, beats per minute) and locomotor activity (Act) responses before and during shaker stress in BPN/3J (grey) and BPH/2J mice (black) at baseline (Baselines pooled from sham and lesion groups). Each dot represents mean ± SEM, averaged across a 30-second period. Bar graphs represent average MAP, HR and locomotor activity response to the stimuli at baseline (B) and average response following sham surgery (S) or MeAm lesion (L). Post sham and lesion values are an average of measurements at 1 and 3 weeks post-surgery. Values are mean ± SEM.